Fundamental Ca\textsuperscript{2+} Signaling Mechanisms in Mouse Dendritic Cells: CRAC Is the Major Ca\textsuperscript{2+} Entry Pathway


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Although dendritic cells (DC) 1 are recognized to play key roles in both initiating and modulating immune responses (1, 2) many fundamental aspects of their biology remain unknown. Ca2+ signaling in DC represents one such area, despite the fact that alterations in intracellular Ca2+ are known to underlie many immune responses. Indeed, a sustained increase in intracellular Ca2+ accompanies T and B cell receptor signaling and is necessary for gene activation, cellular proliferation, and Ab secretion (3, 4).

Similarly, many critical functions in DC appear to involve Ca2+ signaling. Apoptotic body engulfment and processing are accompanied by a rise in intracellular Ca2+ and are dependent on external Ca2+ (5). Chemotactic molecules uniformly produce Ca2+ increases in DC (6-9), suggesting that Ca2+ transients regulate DC migration. DC maturation, including the enhanced expression of MHC class II and costimulatory molecules, is inhibited by chelation of external Ca2+ (10). Conversely, agents that mobilize intracellular Ca2+ can promote DC maturation in the absence of normal cytokine stimulation (10, 11).

However, the Ca2+-signaling pathways involved in these DC functions are not well defined. Chemokine-induced Ca2+ mobilization likely occurs via intracellular inositol trisphosphate (IP3) receptors, because in many cell types the G protein-coupled chemokine receptors are known to activate phospholipase C β2, and in turn, generate IP3. Less is known about Ca2+ entry pathways. Previous studies have suggested the presence of dihydropyridine (DHP)-sensitive Ca2+ channels and ATP-gated channels, although these have not been functionally characterized. Here we have examined Ca2+ entry in DC using electrophysiological and calcium imaging techniques. We show that mouse, myeloid DC express neither functional voltage- nor DHP-gated channels; instead, DHPs mobilize Ca2+ from internal stores. Similarly, ATP signaling leads predominantly to Ca2+ mobilization rather than entry via plasma membrane channels. We show that the major Ca2+ entry pathway in DC is through the Ca2+-release-activated Ca2+ channel (CRAC) (12-14), a plasma membrane channel expressed in many cell types and activated by the depletion of intracellular Ca2+ stores. Furthermore, we show that CRAC is activated during physiologic DC signaling and that activation of CRAC promotes DC maturation.

Materials and Methods

Animals

C57BL/10J (C57) mice, 6-12 wk old, were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center.

Reagents

Recombinant (r) GM-CSF and IL-4 were gifts of S. K. Narula (Schering-Plough, Kenilworth, NJ). Bay K8644, nifedipine, and Na2ATP were obtained from Sigma (St. Louis, MO). Thapsigargin, SKF 96365, and IP3 were obtained from Calbiochem (San Diego, CA).

DC culture and purification

DC were cultured using the method initially reported by Inaba et al. (15) with the following modifications. Bone marrow cells were prepared from...
the femurs and tibias of normal C57 mice and cultured at a density of 3 × 10⁷ cells/ml in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (Nalgene, Miami, FL), nonessential amino acids, t-glutamine, sodium pyruvate, penicillin-streptomycin, and 2-ME (all obtained from Life Technologies). Cultures were supplemented with GM-CSF and IL-4 (at 4 ng/ml and 1000 U/ml, respectively). DC were harvested after a total of 3–4 days of culture for immature cells or 5–6 days of culture for mature cells and purified by metrizamide density (16.5 or 14.5%, respectively) centrifugation. Free [Ca²⁺] in the medium was varied by the addition of a pH-buffered EGTA stock (final concentration 0.4–0.5 mM) or CaCl₂. The actual free [Ca²⁺] with EGTA was verified with a Ca²⁺-selective electrode. The free [Ca²⁺] in normal RPMI 1640 medium (0.44 mM total Ca²⁺) and supplemented medium (5.44 mM total Ca²⁺) was estimated to be ~0.36 and 4.6 mM, respectively, using the software Bound and Determined (16) (available online at http://superior.carleton.ca/~kbstorey).

**Flow cytometry**

Flow cytometric analysis was undertaken using a Beckman Coulter EPICS Elite flow cytometer (Beckman Coulter, Hialeah, FL), and data were analyzed using either WinMDI or EXP32 software (Applied Cytometry Systems, Sheffield, U.K.). Monoclonal Abs specific for MHC class II (IAβ), CD11c, CD80, and CD86 (PharMingen, San Diego, CA) were used as FITC and PE conjugates. Cells stained with species-specific, isotype-matched irrelevant mAbs were used as negative controls. Bone marrow cells cultured for 3–4 days were CD11c⁺, MHC class II⁺, CD80⁺/−, and CD86⁺/−, consistent with immature or "Ag-processing" phenotype reported for DC both in situ or freshly isolated from peripheral tissues. On longer in vitro culture, DC up-regulated their expression of MHC class II Ags and costimulatory molecules consistent with mature DC (15, 17, 18). CD86 expression (vs CD11c) was used to distinguish immature and mature DC in this study (see Fig. 1).

**Fluorescence measurements**

DC were plated on coverslips in culture medium and loaded with Fluo-3AM (3–5 μM) for 20 min at 25 or 37°C degree. Cells were then washed with several volumes of bathing solution and left for another 20 min before recording. Standard bathing solution was (in mM): 130 NaCl, 4 KCl, 10 glucose, 2 CaCl₂, 2 MgCl₂, HEPES, and pH 7.3. Fluorescence measurements were made with either a Zeiss Axiovert 100 TV confocal microscope, or a Deltascan fluorometer (Photon Technology International, South Brunswick, NJ) coupled to a Diastar microscope (Leica, Deerfield, IL). Fluo-3 was excited at 488 nm, and emitted fluorescence was filtered with a 535 ± 25 nm bandpass filter. The fluorescence signal was calibrated in ATP experiments by measuring the maximal fluorescence after treatment with ionomycin (10–50 μM). Absolute estimates of [Ca²⁺] were then obtained by the expression [Ca²⁺] = Keq × (F - Fmin)/(Fmax - F), where Keq is the Ca²⁺ dissociation constant for Fluo3, and Fmax and Fmin are the maximal and minimal fluorescence, respectively. Fmin was assumed to be negligible.

**Electrophysiology**

Whole cell patch clamp recordings were made using an EPC-7 amplifier interfaced to a Macintosh Power PC running IgorPro software (Wavemetrics, Lake Oswego, OR). Patch pipettes with resistances between 2 and 4 MΩ were prepared from aluminosilicate glass (Garner Glass, Claremont, CA). Series resistance compensation was routinely set at ~50%. Data were filtered at 1 kHz and sampled at 5 kHz. For ICRAC recording the bathing solution was (in mM): 145 NaCl, 2 KCl, 2 MgCl₂, 5 Glu, 5 HEPES, and 10 either Ca²⁺/Ba²⁺/Mg²⁺/Sr²⁺. The pipette solution contained (in mM): 2 CsCl, 0.36 and 4.6 mM, respectively, using the software Bound and Determined (16) (available online at http://superior.carleton.ca/~kbstorey).

![Flow cytometry analysis](Image)

**FIGURE 1.** Flow cytometric analysis of immature and mature mouse myeloid DC. Bone marrow cells cultured for 3–4 (A) or 5–6 days (B) were purified by metrizamide density centrifugation then double-immunolabeled for CD11c-FITC and CD86-PE. Histograms, gated for CD11c⁺ DC, show that expression of CD86 (bold) is up-regulated with increased culture. Isotype matched controls are shown (dotted).

![Electrophysiology](Image)

**FIGURE 2.** Absence of voltage- and ATP-activated Ca²⁺ entry in DC. A, Simultaneous voltage-clamp and Ca²⁺ fluorescence recording from a mature DC. A 5-s depolarization from −90 to 0 mV elicited no change in current or [Ca²⁺], rise. The current was recorded using P/4 leak subtraction. No Ca²⁺ currents were detected in a total of 25 cells. B, Application of 200 μM ATP induced a large Ca²⁺ rise in a voltage-clamped DC but no accompanying inward current. In this experiment external Na⁺ was replaced with NMDG to isolate Ca²⁺ currents (see Materials and Methods). C, ATP (20 μM) produced a rapid Ca²⁺ rise in Ca²⁺-rich medium (n = 10). A similar magnitude response was observed in a separate experiment using Ca²⁺-free (0 Ca²⁺/0.2 mM EGTA; with 10 μM contaminating Ca²⁺, this yields ~5 nM free Ca²⁺) medium (n = 10).
128 CsAsp, 10 CsBAPTA, 0.1 CaCl$_2$, 3.16 MgCl$_2$, 10 mM HEPES, pH 7.4.

I CRAC was recorded with 200-ms voltage ramps from $-130$ to $-60$ or $-90$ mV from a holding potential of 0 mV.

For simultaneous patch clamp and fluorescence measurements with ATP and Bay K8644 the bathing solution contained (in mM): 140 N-methyl-D-glucamine chloride (NMDG-Cl), 4 KCl, 10 HEPES, 2 CaCl$_2$, 2 MgCl$_2$, pH 7.3, and the patch pipette contained (in mM): 130 CsCl, 10 NaCl, 10 HEPES, 0.1 EGTA, 4 MgATP, 0.1 GTP, and 50 μM Fluo-3 pentapotassium salt, pH 7.3. Junction potentials of $-10$–$15$ mV (calculated with PClamp software) were corrected offline.

Results

Absence of voltage-, ATP-, or DHP-activated Ca$^{2+}$ entry

To explore immature and mature DC (Fig. 1) for Ca$^{2+}$ entry pathways, we first tested for functional voltage-gated channels. Voltage-clamped cells were depolarized with pulses from $-90$ to 0 mV. In some experiments we also made simultaneous Ca$^{2+}$ fluorescence recordings. Fig. 2A shows that a 5-s depolarization failed to activate an inward Ca$^{2+}$ current or produce any change in intracellular [Ca$^{2+}$]. In summary, no detectable Ca$^{2+}$ currents were observed in either immature or mature DC ($<0.1$ pA/pF, $n = 25$).

Next we tested for ATP-gated ion channels (19, 20), which are expressed in many leukocytes including macrophages (21) and T lymphocytes (22–24). We found that ATP (10–500 μM) produced similar large Ca$^{2+}$ transients in the majority of immature and mature DC tested (109/121). From calibration experiments with the calcium ionophore, ionomycin (see Materials and Methods), we estimated that 100 μM ATP increased free Ca$^{2+}$ to 2.6 ± 0.9 μM ($n = 4$). The ATP-evoked responses desensitized both during ATP application (Fig. 2C) and with repeated applications. In addition, ADP evoked a similar response to ATP (data not shown). Dual ATP/ADP sensitivity is characteristic of the metabotropic P2Y class of receptors (20). Indeed, simultaneous voltage clamping and Ca$^{2+}$ imaging in NMDG$^-$ based medium confirmed that the ATP-

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**FIGURE 3.** DHPs mobilize Ca$^{2+}$ in DC. A. Application of Bay K8644 (15 μM), an L-type Ca$^{2+}$ channel agonist, induced a Ca$^{2+}$ rise in a voltage-clamped DC but no accompanying inward current. B, Nifedipine (50 μM), an L-type Ca$^{2+}$ channel antagonist, induced a similar response in the absence of external Ca$^{2+}$ (0 Ca$^{2+}$/0.2 mM EGTA). The top trace shows the response of a single cell, whereas the bottom trace shows the mean of 10 cells from the same experiment. C, Responses to Bay K8644 in six cells are blocked by depleting intracellular Ca$^{2+}$ stores with thapsigargin (1 μM). These results suggest that DHPs mobilize Ca$^{2+}$ from internal stores by an action independent of L-type Ca$^{2+}$ channels.

**FIGURE 4.** Identification of SOCs in DC using Ca$^{2+}$ fluorescence measurements. The upper trace shows the response of a single DC, whereas the lower trace shows the mean response (±SEM) of 10 DC from the same experiment. Fluo-3-loaded cells were treated with thapsigargin (TG, 1 μM), a microsomal Ca$^{2+}$ ATPase inhibitor, which produced a transient rise in Ca$^{2+}$. Note that application of vehicle, DMSO (0.1%), did not affect intracellular Ca$^{2+}$ levels. The Ca$^{2+}$ rise was solely due to depletion of intracellular Ca$^{2+}$ stores because the bathing solution contained zero Ca$^{2+}$/0.2 mM EGTA. The reapplication of Ca$^{2+}$-rich bathing solution produced a larger sustained Ca$^{2+}$ increase. Similar responses were seen in a total of 63 DC.
evoked Ca$^{2+}$ rise was largely independent of inward Ca$^{2+}$ current (Fig. 2B). In some experiments small inward currents were observed (20 ± 4 pA, n = 5), but these currents occurred at variable times following ATP application and did not always correlate with the Ca$^{2+}$ transient. Furthermore, we found that the magnitude of ATP-induced Ca$^{2+}$ rises was unaffected when Ca$^{2+}$ was removed from the external medium (Fig. 2C; control 277 ± 20%, n = 24, zero Ca$^{2+}$, 275 ± 28%, n = 18), although the duration was considerably shortened (τ = 89 ± 20 s vs τ = 22 ± 3 s, p < 0.01) due to elimination of a late Ca$^{2+}$ plateau or hump. This Ca$^{2+}$ hump may be the result of store-operated Ca$^{2+}$ entry as described below. These results suggest that mouse myeloid DC predominantly express metabotropic purinoceptors, which mobilize Ca$^{2+}$ by formation of IP$_3$ and not via plasma membrane ATP-gated ion channels. This is consistent with the recent report of functional P2Y type receptors in human myeloid DC (25).

Next, we examined whether a presumed voltage-insensitive L-type Ca$^{2+}$ channel, recently identified in human myeloid DC (26), was present in mouse myeloid DC. Similarly we found that 10–25 μM Bay K8644 (an L-type channel agonist) evoked large Ca$^{2+}$ rises (Fig. 3A, n = 20). However, inward currents did not accompany these Ca$^{2+}$ rises. In addition, we found that nifedipine (an L-type channel antagonist) also elicited Ca$^{2+}$ rises, and these Ca$^{2+}$ responses persisted in Ca$^{2+}$-free medium (Fig. 3B). In contrast, pretreatment with thapsigargin to deplete Ca$^{2+}$ stores occluded the Bay K8644-evoked response (Fig. 3C). These results indicate that 1,4 DHPs do not induce Ca$^{2+}$ entry through a plasmalemmal channel, but rather mobilize Ca$^{2+}$ from internal stores.

**Store-operated Ca$^{2+}$ entry**

The presence of store-operated channels (SOCs) in DC was investigated by treatment of Fluo-3-loaded cells with the microsomal Ca$^{2+}$-ATPase inhibitor, thapsigargin. This is a commonly used method for activating SOCs in other cell types (4, 27). In Fig. 4A, the upper trace shows the Ca$^{2+}$ fluorescence of a single cell, whereas the lower trace shows the mean fluorescence of 10 cells from the same experiment. Application of thapsigargin in 10 nM bathing Ca$^{2+}$ (0.2 mM EGTA and no added Ca$^{2+}$) produced a small increase in cytosolic Ca$^{2+}$ due to depletion of internal stores, and this slowly declined over 3 min. Reapplication of 2 mM external Ca$^{2+}$ induced a large and sustained increase in intracellular Ca$^{2+}$. Similar responses to thapsigargin treatment were observed in the majority of immature (38/39) and mature (25/26) DC tested. No responses were seen when cells were incubated in zero Ca$^{2+}$ without thapsigargin. This dependence of Ca$^{2+}$ entry on prior Ca$^{2+}$ mobilization suggests that SOCs mediate the entry. We tested several common pharmacological blockers of SOCs. SKF 96365 (10 μM) completely inhibited the response (n = 20), as did...
1 mM Cd\(^{2+}\) \((n = 20)\). In contrast, 100 \(\mu\)M Cd\(^{2+}\) \((n = 10)\), a concentration sufficient to inhibit voltage-gated Ca\(^{2+}\) channels but not SOCs, and nimodipine \((n = 10)\), a specific L-type Ca\(^{2+}\) channel blocker, had no significant effect (data not shown). These results suggest that the Ca\(^{2+}\) influx following thapsigargin treatment was via SOCs.

To investigate the properties of these SOCs further, and to determine whether these channels carried a nonspecific cation current or alternatively a Ca\(^{2+}\) selective current \(I_{\text{CRAC}}\), we conducted whole-cell voltage clamp experiments. In these experiments Ca\(^{2+}\) stores were depleted by the inclusion of IP\(_3\) together with the Ca\(^{2+}\)-chelator BAPTA in the patch pipette solution. Fig. 5A shows the whole cell currents elicited by voltage ramps from \(-120\) to \(+100\) mV in the presence of external solutions containing different divalent cations. In Fig. 5B (from the same experiment as in Fig. 5A) the \(I_{\text{CRAC}}\) develops after break-in. The current is robust and reproducibly altered with different divalent cations. Inward currents were activated at hyperpolarized potentials with either 10 mM Ca\(^{2+}\), Ba\(^{2+}\), or Sr\(^{2+}\). Most of the inward current was inhibited when Mg\(^{2+}\) replaced Ca\(^{2+}\). The remaining inward current and the outward current in Mg\(^{2+}\) most likely represents leak current. Subtracting this leak current from the other currents revealed the pure \(I_{\text{CRAC}}\). Consistent with \(I_{\text{CRAC}}\) in other cells \((12, 28)\) this current exhibits a characteristic inward rectification \((\text{inset})\). The relative conductivity was Ca\(^{2+}\) > Ba\(^{2+}\) > Sr\(^{2+}\) with Ca\(^{2+}\) conductivity roughly 2-fold greater than Ba\(^{2+}\) and Sr\(^{2+}\). A similar permeability sequence was reported for \(I_{\text{CRAC}}\) in T lymphocytes \((27)\). The Ca\(^{2+}\) current density at \(-80\) mV was \(-0.7\) pA/pF \((n = 3)\) and again is similar to values reported for T lymphocytes of \(-1\) pA/pF \((12, 29)\).

\(I_{\text{CRAC}}\) is known to be highly selective for divalent over monovalent cations. In agreement with this, we found that \(I_{\text{CRAC}}\) in DC was essentially independent of the external [Na\(^{+}\)] (Fig. 6A). In addition, the current was reversibly inhibited by 1 \(\mu\)M SKF 96365 (Fig. 6B). This inhibition by SKF 96365 is consistent with the block of Ca\(^{2+}\) entry observed in our imaging experiments. These results indicate that SOC in DC are Ca\(^{2+}\)-selective channels and similar to the channels that mediate \(I_{\text{CRAC}}\).

CRAC is activated during ATP signaling

We next considered whether CRAC is activated under physiological conditions. ATP is a putative DC chemotactic factor \((25)\) and may attract DC to sites of cell injury and inflammation and stimulate DC maturation \((30, 31)\). ATP-evoked Ca\(^{2+}\) responses exhibited two components: a fast Ca\(^{2+}\) transient that was independent of external Ca\(^{2+}\) and a slower Ca\(^{2+}\)-dependent plateau (Fig. 2C). This slower ATP-evoked Ca\(^{2+}\) response suggests that a SOC may be activated in DC during purinergic receptor signaling. To explore this further we studied the dependence of this delayed ATP-induced Ca\(^{2+}\) entry on both external Ca\(^{2+}\) and voltage. In these experiments DC were either perfused with the standard saline solution \((4\, \text{mM K}^{+})\) to generate a normal resting potential of \(-50\) to \(-60\) mV, or a solution containing 140 mM K\(^{+}\) to clamp the membrane potential close to 0 mV. In separate experiments where DC were held under current-clamp, we confirmed that high K\(^{+}\) does not alter the ATP-evoked Ca\(^{2+}\) transient.

FIGURE 6. \(I_{\text{CRAC}}\) in DC is Na\(^{+}\) independent and blocked by SKF 96365. Currents were recorded in response to 200-ms voltage ramps from \(-110\) to \(+50\) mV. A, \(I_{\text{CRAC}}\) was similar in Na\(^{+}\)-containing or Na\(^{+}\)-free (Na\(^{+}\) replaced with NMDG) bathing solutions. Cell capacitance was 18 pF. B, \(I_{\text{CRAC}}\) recorded from a different cell \((C = 30\, \text{pF})\) was reversibly inhibited by 1 \(\mu\)M SKF 96365.

FIGURE 7. ATP signaling in DC activates Ca\(^{2+}\) entry with properties similar to CRAC. ATP \((100\, \mu\)M\) evoked a rapid Ca\(^{2+}\) transient in a fluo-3-loaded DC in Ca\(^{2+}\)-free solution. Readdition of 2 mM Ca\(^{2+}\) medium evoked a Ca\(^{2+}\) rise in standard \((\text{low KCl})\) saline but not when the medium contained 140 KCl used to depolarize the cell to \(-0\) mV (see text). Thus, the voltage dependence of this Ca\(^{2+}\) rise is consistent with activation of CRAC.
depolarize cells to \(-\sim 0 \text{ mV}\). This dependence of membrane potential on external \([K^+]\) may arise from the presence of leak or voltage-activated \(K^+\) currents (32). Fig. 7 shows that application of 100 \(\mu\text{M}\) ATP in \(Ca^{2+}\)-free medium evoked a rapid \(Ca^{2+}\) transient in a DC. The readaptation of \(Ca^{2+}\) induced a smaller, long lasting \(Ca^{2+}\) rise but only when the DC was held at a negative resting potential; no \(Ca^{2+}\) rise was seen when the cell was depolarized to \(0 \text{ mV}\), and no \(Ca^{2+}\) rise was seen when cells were incubated in zero \(Ca^{2+}\) (up to 3 min) without ATP \((F/F_0 = 0.98 \pm 0.04, n = 20)\), ruling out the possibility that CRAC was activated by a passive loss of \(Ca^{2+}\). In a total of 10 cells, ATP evoked a normalized \((F/F_0)\) \(Ca^{2+}\) plateau of \(1.3 \pm 0.2\). The voltage dependence of the delayed ATP-evoked \(Ca^{2+}\) entry is consistent with it being mediated by CRAC.

**Activation of CRAC induces phenotypic maturation of DC**

Next we assessed whether CRAC participates in DC maturation. A heterogenous culture of immature and mature mouse myeloid DC were incubated overnight (18 h) with 50 nM thapsigargin to activate CRAC, and then double immunostained as described in Materials and Methods to detect their surface expression of MHC class II and costimulatory molecules. Fig. 8 shows that untreated controls were a heterogenous mix of immature (MHC class II \(^{\text{dim}}\), CD80 \(^{\text{dim}}\), CD86 \(^{\text{dim}}\)) and mature (MHC class II \(^{\text{high}}\), CD80 \(^{\text{high}}\), CD86 \(^{\text{high}}\)) DC. In contrast, DC exposed to thapsigargin overnight were homogenously mature, and expressed high levels of MHC class II, CD80, and CD86. These results complement those previously described for thapsigargin in myeloid leukocytes, including transformed cell lines, monocytes, and cultured bone marrow cells (10). Thapsigargin also mobilizes \(Ca^{2+}\) from intracellular stores. To test that stimulation by thapsigargin depended on \(Ca^{2+}\) entry via CRAC, we repeated the experiment in a low \(Ca^{2+}\) medium (1 \(\mu\text{M}\) free); however, under these conditions DC viability was reduced by \(-\sim 50\%\). As an additional test of the involvement of CRAC in DC maturation, we cultured DC overnight in medium containing different free \(Ca^{2+}\) concentrations (0.001, 0.36, and 4.6 mM) without any other stimuli. Because our results above indicate that CRAC is the major \(Ca^{2+}\) entry pathway, then varying the transmembrane \(Ca^{2+}\) gradient should mainly affect current via CRAC. Fig. 9 shows that the percentage of CD86\(^{+}\) DC (right-hand peak) increased in direct proportion to \([Ca^{2+}]\). Thus, this result suggests that modulating the passive \(Ca^{2+}\) entry via CRAC is capable of influencing the spontaneous, in vitro maturation of DC.

**Discussion**

This study constitutes the first in-depth investigation of \(Ca^{2+}\) signaling in DC. Our results demonstrate that mouse myeloid DC (both immature and mature) express SOCs with the properties of CRAC, but express neither functional voltage-dependent \(Ca^{2+}\) channels nor DHPR-gated channels and few if any ATP-gated ion channels. Importantly, this suggests that CRAC is likely to be the major \(Ca^{2+}\) entry pathway in DC and thus an intrinsic component of the \(Ca^{2+}\) signaling processes that drive DC maturation and migration. The absence of voltage-dependent channels is not surprising given that myeloid DC, like other leukocytes, are essentially nonexcitable, and voltage-gated \(Ca^{2+}\) channels have not been clearly demonstrated in leukocytes (4). In contrast, ATP-gated ion channels are expressed in macrophages (closely related myeloid-lineage cells) (21), mast cells (33), and T cells (22–24). ATP-gated channels are \(Ca^{2+}\) permeable. In macrophages they are important in lipopolysaccharide-activated inflammatory responses (34, 35), in mast cells they modulate histamine secretion (33), and in T lymphocytes they may play a role in cell differentiation (24) or death (22, 23). Our results indicate that ATP signaling in DC predominantly occurs via the metabotropic (P2Y) class of receptors. In support of this, ATP responses were recorded without accompanying membrane current and in zero external \(Ca^{2+}\), and similar responses were seen with ADP. These results agree with a previous patch-clamp study using human myeloid DC (25). Several types of P2Y receptor have been identified in human DC (30, 36). The P2Y receptor is structurally similar to the chemokine receptor family; both are seven-transmembrane G protein-coupled receptors.
receptors and they share identical intracellular signal transduction cascades (37). Therefore, P2Y-mediated signaling may contribute to chemotaxis, attracting DC to sites of inflammation. Consistent with this idea, ATP has been shown to alter DC shape and dendrite orientation (25). Although mRNA for the P2X channels (P2X1,4,5,7) have been identified in DC by RT-PCR (30, 36, 38) functional evidence for channel expression is weak. For example, although Berchtold et al. observed that ATP (100 μM) evoked rapid Ca2+ rises, these were not affected by the buffering of external Ca2+. In contrast, there are reports that high ATP concentrations (0.75–5 mM) can permeabilize DC to low molecular mass dyes (36, 38, 39), indicating functional P2X7 channels. The high concentrations required may explain why no P2X7 currents were seen here with 10–500 μM ATP. Whether millimolar intracellular ATP levels play a biological role in DC function is unclear. Moreover, the fact that large Ca2+ transients can be evoked by low ATP (~10 μM) via P2Y receptors suggests strongly that this latter pathway is more physiological. It is also significant that DC express high levels of membrane ATPase activity (30, 40) and that extracellular ATP is very rapidly hydrolyzed by DC (41). Thus, this would limit the activation of P2X7 channels and serve to protect DC, relatively rare leukocytes, from ATP-induced apoptosis (22, 23, 41).

An important feature of this study is that we have clarified whether DC express functional DHP-gated channels. Poggi et al. (26) reported that human myeloid DC express the β subunit of L-type Ca2+ channels and that the DHP, Bay K8644, but not membrane depolarization, induced increases in intracellular [Ca2+]i. These data were interpreted as evidence for the presence of non-voltage-gated L-type Ca2+ channels. In this study we found that Bay K8644 could indeed produce Ca2+ increases in mouse myeloid DC. However, we further found that these Ca2+ increases were independent of external Ca2+ and changes in membrane conductance. These data are not inconsistent with Poggi et al. because they did not report whether Bay K8644 responses were dependent on external Ca2+. Our data clearly indicate that DHP-induced Ca2+ rises are not due to Ca2+ entry but rather the result of Ca2+ mobilization from internal stores. Accordingly, we found that responses to DHPs were occluded by emptying intracellular Ca2+ stores with thapsigargin. Moreover, we found that Ca2+ mobilization was also induced by nifedipine, an L-type channel antagonist. Thus, this strongly suggests that DHPs do not act via L-type channels. Just how DHPs mobilize Ca2+ is unclear. Nevertheless, the signaling pathway deserves further attention because nifedipine has been found to modulate numerous DC functions, including inhibition of Ag processing (42), apoptotic body engulfment, and IL-12 secretion (26).

The biophysical properties of CRAC in DC are similar to those reported in mast cells and Jurkat T cells (12, 29, 43). The relative conductance sequence is Ca2+ > Ba2+ ≃ Sr2+ ≃ Mg2+ with Ca2+ permeability approximately twice that of Ba2+ and Sr2+. The Ca2+ current density at −80 mV is ~0.7 pA/pF, similar to that in Jurkat T cells (~1 pA/pF). The current exhibits inward rectification such that the current at −60 mV is 3- to 4-fold larger than at 0 mV (Fig. 5A), more than what would be expected from the difference in driving force. Again this is similar to that reported in T cells (44). Thus, modulation of resting membrane potential is likely to have marked effects on the degree of Ca2+ entry. Interestingly, the resting membrane potential of human myeloid DC becomes more hyperpolarized following activation with TNF-α (P.J.O. and G.P.A., unpublished observations), and this may serve to augment Ca2+ entry through SOCs.

It is notable that store-operated Ca2+ entry was observed in the majority of both immature and mature DC, indicating its importance in a range of DC functions. Ca2+ signaling is involved in DC maturation, chemotaxis, and migration to secondary lymphoid tissue (6–9), thus, it is likely that CRAC plays an important role in all these processes. Our data are consistent with this proposal. First, ATP, a putative physiological activator of DC, induced store-operated Ca2+ entry with properties similar to CRAC (Figs. 2C and 7). Second, activation of CRAC with thapsigargin induced marked DC maturation (Fig. 8). This finding in mouse myeloid DC is consistent with the previously reported observations for thapsigargin using human myeloid cell lines, monocytes, and DC (10, 11). Third, spontaneous DC maturation was directly proportional to the extracellular Ca2+ concentration (Fig. 9). Thus, a CRAC signaling pathway is likely to be involved in DC maturation. On a practical note, these results suggest that it may be useful to supplement DC culture medium (such as RPMI 1640), containing only 0.4 mM total Ca2+, with extra Ca2+ to optimize maturation.

In summary, this study has shown that a SOC with properties similar to CRAC plays a dominant role in DC Ca2+ signaling, with little contribution from the other Ca2+ entry pathways that are common in leukocytes.

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